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Citation for final published version:

Paziewska-Harris, A. ORCID: <https://orcid.org/0000-0001-9677-4753>, Schoone, G. and Schallig, H. D. F. H. 2016. An easy 'one tube' method to estimate viability of *Cryptosporidium* oocysts using real-time qPCR. *Parasitology Research* 115 (7) , pp. 2873-2877. 10.1007/s00436-016-5044-z file

Publishers page: <http://dx.doi.org/10.1007/s00436-016-5044-z>  
<<http://dx.doi.org/10.1007/s00436-016-5044-z>>

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An easy 'one tube' method to estimate viability of *Cryptosporidium* oocysts using real-time qPCR.

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**Abstract** Viability estimation of the highly resistant oocysts of *Cryptosporidium* remains a key issue for the monitoring and control of this pathogen. We present here a simple 'one tube' qPCR protocol for viability estimation using a DNA extraction protocol which preferentially solubilizes excysted sporozoites rather than oocysts. Parasite DNA released from excysted sporozoites was quantified by real-time qPCR using ribosomal DNA marker. Using the method, the qPCR signal was directly proportional to the number of oocysts excysted, and a power law relationship was noted between oocyst age and the proportion excysting. Unexcysted oocysts released negligible amounts of DNA making the method suitable for estimating viability of as few as 10 oocysts.

#### KEYWORDS

*Cryptosporidium parvum*; excystation; sporozoites; environmental samples; water quality monitoring

## Introduction

The apicomplexan genus *Cryptosporidium*, parasitic in the stomach and intestine of vertebrate hosts, includes several pathogens of humans, the most important of which are the zoonotic *C. parvum* and the human-specific *C. hominis*, although sporadic infections with other species have also been described (Xiao 2010). The dispersive oocysts are shed by infected hosts in large numbers into the environment (see e.g. Nydam et al. 2001, Chappell et al. 2006), where they contaminate soil and water and pose a threat for human and animal health. The oocysts are highly resistant and easily dispersed, and a dose of only 10 may be infective (Zambriski et al. 2013). There is no effective treatment and the disease can be fatal for the immunocompromised. Much time and money is therefore expended to detect, control and eliminate *Cryptosporidium* contamination of drinking water; for example most recently, in Lancashire, UK in August 2015, 300 000 households were advised to boil drinking water for over a week because the parasite had been detected in a water treatment plant. A key issue for *Cryptosporidium* monitoring is that of establishing the viability and infectivity of any oocysts found (King and Monis 2007). The gold standard should be to measure the potential of oocysts to initiate infections in living hosts, but this is of course impossible under most circumstances, and viability is most often assessed by microscopical assays such as vital dye-exclusion (e.g. Campbell et al. 1992; Jenkins et al. 1997) or in-situ hybridization based on the persistence of RNA in living oocysts (Smith et al. 2004), or by bulk assays such as excystation rate (Connelly et al. 2007), RNA content estimated by reverse transcription (RT) PCR (Garcés et al. 2006) or infectivity for host cell cultures, sometimes monitored by PCR (Shahidauzzaman et al. 2009). Quantitative PCR (qPCR) approaches are highly sensitive and avoid the complication of multiple handling steps with associated loss of material, which can be a disadvantage when working with small numbers of oocysts as may be expected in water monitoring, but of course fail to establish viability. Here we describe a simple method for viability estimation, achieved by measuring DNA concentration after a simple, one tube excystation-DNA extraction step. Coupled with real-time qPCR the method allows reliable, accurate estimation of the proportion of living oocysts in samples, and is capable of application to samples of as few as 10 purified oocysts.

## Methods

### Oocysts and pre-treatment

*Cryptosporidium parvum* IOWA strain oocysts (Waterborne, Inc.) stored in phosphate-buffered saline (PBS) supplemented with 100U/ml penicillin, 100 µg/ml streptomycin, 10 mg/ml gentamicin, 0.25 µg/ml Amphotericin B and 0.01% Tween 20 at 4°C were used in all experiments. Oocysts of different age post-purchase from Waterborne Inc. (45, 75, 90, 160, 190 and 300 days old) were used to establish a range of samples of different viabilities. The concentration of supplied oocysts was initially calculated using a hemocytometer (W. Schreck, Hofheim/TS, Germany), and samples of different oocyst concentration (10, 50, 100, 1000, 5000 and 10000 oocysts) generated by dilution. The standard excystation protocol (Hijjawii et al. 2001) consisted of incubation of oocysts in a 1.5 ml reaction vial (Eppendorf) for 30 minutes at 37°C in a 0.25% trypsin (from bovine pancreas, Sigma, cat. no. T1426) solution adjusted to pH 2.5 with 1M hydrochloric acid, followed by centrifugation at 2000 × g (bench top centrifuge) for 5 minutes. The trypsin solution was replaced by 200 µl of *Cryptosporidium* maintenance medium (Hijjawi et al. 2001, modified as in Paziewska-Harris et al. 2015) containing 200 µg/ml of bile salts (from bovine and ovine pancreas, Sigma, cat. no. B8381) and incubated for a further 2.5 hours at 37°C. Lysis buffer (1 ml; Boom et al. 1990) was added to the samples which were stored at 4°C for up to 2 days before DNA extraction. All steps were carried out in the same reaction vial, and incubations were performed using heat blocks. Simultaneous with every batch of excysted oocysts, a similar control batch of oocysts, which had not been excysted but

which had experienced a dummy incubation in 200 µl distilled water, followed by centrifugation and incubation in a second aliquot of distilled water was processed.

## DNA isolation

DNA isolation used a modified technique described by Boom et al. (1990). Following addition of 1 ml of the guanidinium thiocyanate based L6 buffer (120 g GuSCN dissolved in 100 ml 0.1 M Tris hydrochloride [pH 6.4] and mixed with 22 ml 0.2 M EDTA [pH8.0] and 2.6 g Triton X-100) to the oocyst sample, 40 µl of silica (Sigma) size-fractionated as described by Boom et al. (1990) was added and the tube shaken for 5 minutes. Subsequently it was centrifuged at 14000 rpm for 30 s, and the supernatant discarded. One ml of wash buffer L2 (120 g of GuSCN dissolved in 100 ml of 0.1 M Tris hydrochloride [pH 6.4]) was added to the tube, the contents were mixed by inversion and centrifuged again at 14000 rpm for 30 s. Following further rinses with 1 ml of 70% ethanol and 1 ml of acetone, with intervening centrifugations (14000 rpm for 30 s), the silica was air-dried and 50 µl of sterile water added to the tubes, after which the samples were incubated for 5 min in 56°C to elute DNA. After brief centrifugation (14000 rpm for 30 s), eluted DNA was transferred to a new tube and stored at -20°C prior to use in real-time qPCR.

## PCR conditions

Extracted DNA was used as previously described (Paziewska-Harris et al. 2015) in a PCR assay targeting a 318 bp fragment of 18S rDNA of *Cryptosporidium parvum* (bases 453-770, Genbank accession L16996). Each amplification reaction consisted of 1× concentrated VeriQuest mix (Affymetrics, USA), 0.25 µM of each primer (forward: 5'- GAA ATA ACA ATA CAG GAC TTT TTG GTT TTG-3', reversed: 5'- TTA TTC CAT GCT GGA GTA TTC AAG GCA TAT-3'; Biolegio, The Netherlands), 0.12 µM probe (5' FAM [6-carboxyfluorescein]-TAC GAG CTT TTT AAC TGC AAC AA-XS-BHQ[Black Hole Quencher] 3'; Biolegio, The Netherlands) and 1.25 µl of DNA in 12.5 µl of final volume. With each set of reactions a negative control (sterile water) and a standard curve for quantification of the parasites were used. The standard curve was based on DNA isolated from *Cryptosporidium* sporozoites. The physical concentration of oocysts and sporozoites was calculated by pipetting 10 µl of oocysts or freshly-excysted sporozoite suspension between the counting chamber and cover slide of a hemocytometer (W. Schreck, Hofheim/TS, Germany). After a 5-minute settlement period, parasites were counted using an inverted microscope at 100× magnification. A total of 25 0.004 mm<sup>3</sup> squares of the chamber were counted, giving a total counting volume of 0.1 mm<sup>3</sup> (=0.1 µl). After multiplying by 10, the concentration of parasites/µl was obtained, and a 10-fold dilution series prepared from 8000 parasites/µl down to 0.08 parasites/µl. The thermal profile of the PCR reaction was: 50°C for 10 min (for Uracil-DNA glycosylase activation), 95°C for 10 min, 50 cycles of 95°C for 15 s and 60°C for 1 min, with a plate read after each cycle, and the reactions were performed in CFX96 Touch™ real-time PCR blocks (BioRad, USA).

## Results

The PCR assay was able to detect between 0.4 and 4 copies of 18S rDNA/µl (Fig. 1), which translates to a concentration of between 0.08 and 0.8 sporozoite per µl of DNA elution buffer, given that each sporozoite contains 5 copies of 18S rDNA (Abrahamsen et al. 2004). The signal in qPCR for 4 copies of 18S rDNA/ µl was obtained with a frequency of 92% with Ct (cycles to threshold) value of 36.08 to 38.83, while the signal from 0.4 copies was obtained in 29% cases with a Ct value of 36.21 to 38.54. On all graphs the estimated number of oocysts has been calculated on the basis that one oocyst contains 4 sporozoites. The standard curve shows a sensitivity of the qPCR for as few as 1 sporozoite in the PCR reaction. At the lowest DNA concentration, the lowest threshold cycle for the qPCR is

around cycle 36-39, and at this concentration the reaction worked in 7/24 reactions, consistent with the inclusion of a template DNA copy in 30% of all reactions.

Oocysts exhibit a sharp decline in viability, as measured by their ability to excyst, after storage at 4°C. Following 45 days storage, 95% of oocysts remain able to excyst, but there is then a rapid decline in excystation rate to 14% for the 75-day-old oocysts (see Fig. 2a). This loss of viability can be modelled by a power curve (the best model indicated by Bayesian information criterion [BIC]):

where  $y$  = estimated number of oocysts and  $x$  = age of oocysts, with a linear correlation coefficient ( $R^2$ ) for the log transformed equation of 0.73 (Fig. 2b). Estimation of the DNA concentration of unexcysted control oocysts, which had not been pre-treated with trypsin and bile salts, was between 0.1-1.8% of the original input, regardless of the age of oocysts (Fig. 2c).

For oocysts stored at 4°C for 60 days and then excysted, the relationship between the number of inputted oocysts and qPCR-based DNA estimation was linear (choice of the model based on BIC), with an  $R^2$  of 0.875 (Fig. 3a). Based on this linear relationship, the proportion of oocysts releasing viable sporozoites was predicted by the equation

where  $y$  = the number of emerging sporozoites and  $x$  = the input dose of oocysts, suggesting a viability for the oocysts of c. 31%, compared to a predicted viability of 30% from Fig. 2b. For oocysts which had been stored for 75 days, the fitted equation

had an  $R^2$  of 0.94 (Fig. 3a); the coefficient of 0.175 suggests a viability of 17%; the predicted viability of these cysts (Fig. 2b) was 14%. In all cases however, the viability estimated from individual replicates varied between 35% and 65% for the 60d old oocysts and 8% and 31% for the 75d oocysts. The signal from unexcysted oocysts corresponded to a sporozoite yield ranging from 0 for the lowest input up to 100 for the input of 10000 60-day old oocysts, and up to 600 for an input of 10000 75-day cysts (Fig. 3a,b).

## Discussion

The current methodology represents a one-tube approach to estimate small numbers of oocysts with minimal wastage, and could detect DNA from viable *Cryptosporidium* oocysts in samples of as few as 10 oocysts. The sensitivity of the reaction was at least as good as that shown for RT-PCR by Guy et al. (2003), and represents as sensitive a detection methodology for living *Cryptosporidium* as any currently available. The key step is the excystation of oocysts and the extraction of sporozoite DNA within a single tube, avoiding the need for either the extraction of DNA from oocysts, or the conversion of extracted RNA to cDNA (Guy et al. 2003), both methodologies requiring high efficiency extraction of nucleic acids from oocysts. The high sensitivity and one-tube protocol strongly recommend the method for the evaluation of *Cryptosporidium* viability based on small cyst counts, such as are obtained from water or soil samples. The detection of DNA from excysted sporozoites from small samples (100 or 10 oocysts) was stochastic, but this will always be an issue with very small numbers of oocysts; within samples, viable oocysts must be present, and when present, the DNA from the excysted sporozoites must be present in the PCR reaction. We recommend that when estimating viability from as few as 100 or 10 oocysts, the number of PCR replicates performed on the

template be correspondingly increased, to allow viability to be estimated according to the Poisson distribution of positive reactions.

Testing of the method on the oocysts of different age gave the results similar with those observed by Liang and Keely (2012) based on mRNA analysis, where oocysts stored for 3 months at 4°C in PBS showed just under 35% viability as compared to the fresh ones, and after 11 months the loss of viability increased to 99% (compared to 99.5% after 10 months as estimated in our study). This suggests that the method of estimating viability described in this study can be used with similar precision as methods based on the RNA analysis.

The current methodology also highlights the great problem with nucleic acid extraction from *Cryptosporidium*; the difficulty experienced in solubilizing the oocyst. The current methodology uses the Boom protocol (Boom et al. 1990) for nucleic acid extraction, which is based on the solubility of proteins in guanidinium thiocyanate (GuSCN). Guanidinium thiocyanate is a potent chaotropic agent capable of solubilizing almost any protein substrate, and the failure of oocysts to dissociate in the extraction buffer in this methodology is probably connected with specific composition of outer layers of oocyst wall (glycocalyx and lipid hydrocarbon; see e.g. Jenkins et al. 2010 for details), which are not disrupted by GuSCN. Furthermore, the Boom et al. (1990) protocol does not include a reducing agent (e.g. 2-mercaptoethanol) with the guanidinium reagent, and possibly for this reason the lysis buffer is unable to break down disulphide bonds within the protein component of the oocyst cell wall, which adds to its resistance. Instead, intact unexcysted oocysts are removed during the silica-binding and subsequent washes of the Boom (1990) protocol, and the final extracted DNA is derived only from excysted sporozoites, with at most 1% of the DNA template being derived from unexcysted oocysts (Fig. 3). Commercial column methodologies which include reducing agents may not work in this same manner, and the vagaries of local concentration of reductants in, for example, faecal extractions, could lead to considerable errors in *Cryptosporidium* oocyst estimation based on total DNA. At the same time, commercial kits elute nucleic acids in larger volumes, and often do not employ a single tube. In the current protocol, the elution volume could be reduced when dealing with particularly small samples.

A further issue with the methodology concerns an apparent decline in viability/sporozoite abundance at the highest oocyst concentrations. This does not appear to be an issue with DNA binding because the highest standard on the standard curve utilized a similar oocyst number than the maximum number excysted in the experimental series. One possibility is that parasite DNA binds to glycoproteins (Liu et al. 2010) in oocyst fragments in the medium, potentially reducing total apparent DNA abundance at the highest oocyst concentrations. However, since the method is designed for, and is most effective with very small oocyst numbers, in practice this saturation effect should not be important.

The current method is several orders of magnitude more sensitive than microscopy, and much less labor-intensive, requiring far fewer washing and staining steps. It is also a more relevant measure of viability; it measures the success of a vital process, excystation, rather than a proxy such as energy supply or mRNA pools. Being based in a single reaction vial with minimal washes to cause oocyst loss, it is simple and capable of incorporation into routine screening protocols for *Cryptosporidium* in environmental samples. Linked to, for example, magnetic bead technology to isolate and concentrate oocysts, we envisage the method providing a significant improvement in assessment of viability of *Cryptosporidium* recovered from a variety of sources including ground water, soil and animal faeces.

**Conflict of Interest:** The authors declare that they have no conflict of interest.

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**Fig. 1** Standard curve for qPCR targeting *Cryptosporidium* 18S rDNA based on a 10-fold dilution series of 40000 copies. Linear regression ( $y = -1.272\ln(x) + 37.325$ ,  $R^2 = 0.997$ ) shown as dashed line.

**Fig. 2** Comparison of oocysts of different ages; a) mean number of oocysts excysting (initial number treated = 8650) following trypsin/bile salts pre-treatment, estimated by qPCR performed on DNA obtained from the total sample; b) decline in excystation success of trypsin/bile salt pre-treated oocysts, based on qPCR of DNA from the total sample, plotted on a logarithmic scale. Regression (dashed line) follows the equation  $y = 2 \times 10^8 \times x^{-2.747}$  ( $R^2 = 0.73$ ); c) estimation of total oocyst number (without pre-treatment). Note that the logarithmic scale is one order of magnitude smaller than in (b), and the variability of DNA estimation due to inefficiency of extraction from oocysts.

**Fig. 3** Comparison of the number of oocysts excysting based on qPCR-estimation of *Cryptosporidium* DNA from a) trypsin/bile salt pre-treated (circles) and untreated (diamonds) 60-day (open markers, dashed trendline) and 75-day (solid markers, solid trendline) oocysts; equations for the trendlines given in text; b) 60-day (open columns) and 75-day (solid columns) old oocysts liberating DNA (mean  $\pm$  SD, based on minimum of 3 replicates) without pre-treatment to stimulate excystation. Note the difference in scale between (a) and (b).

Figure 1

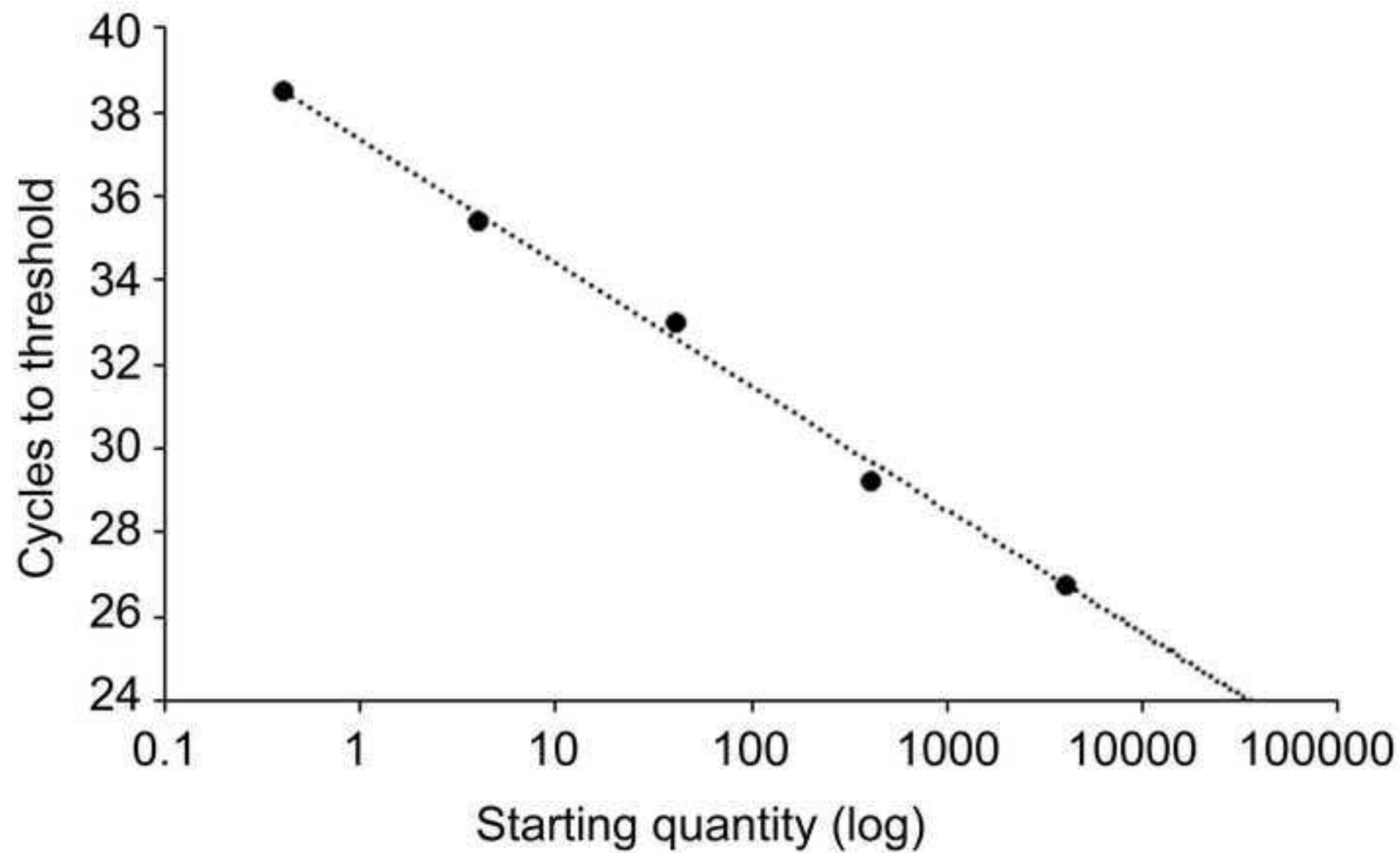


Figure 2

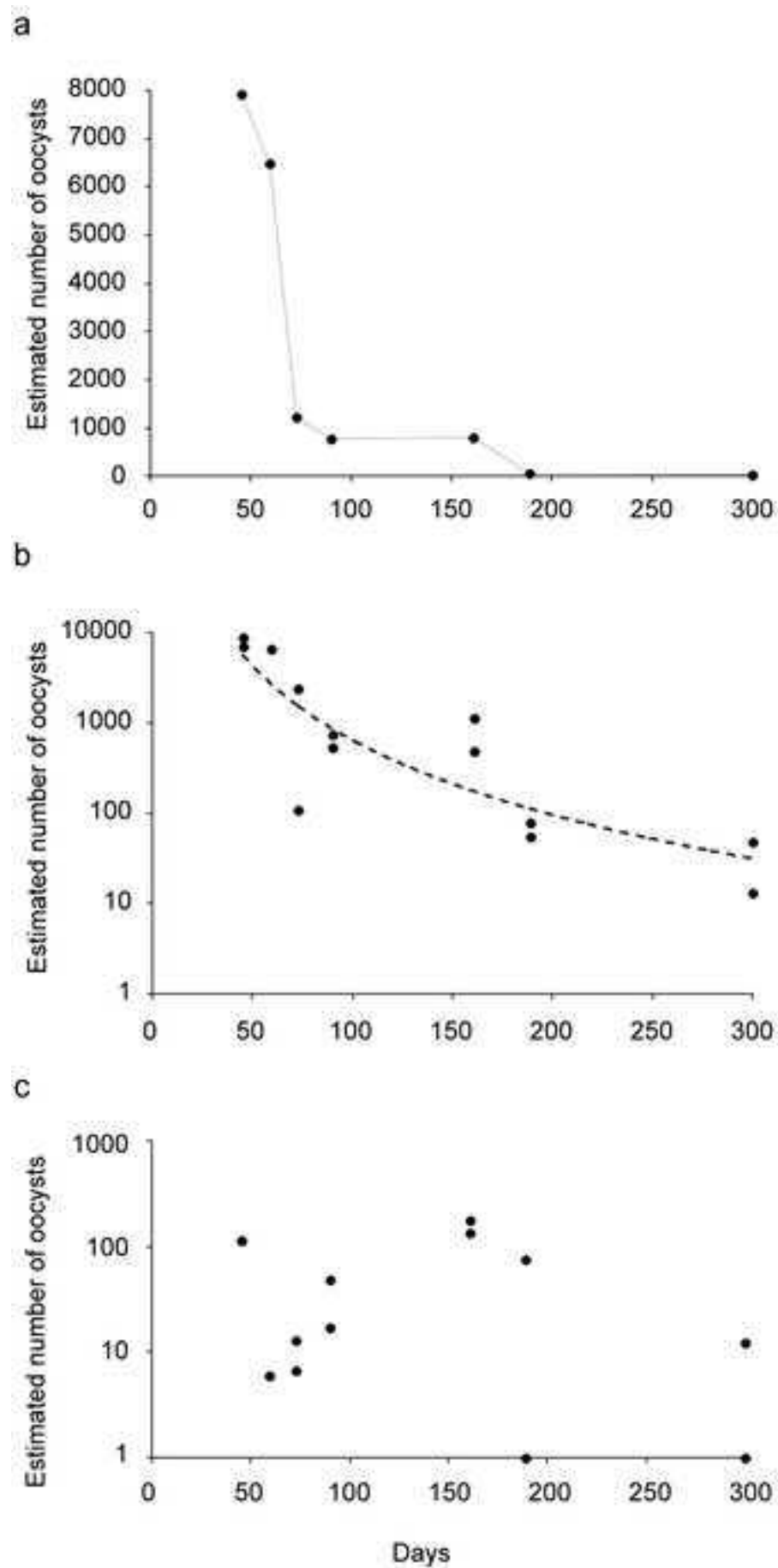
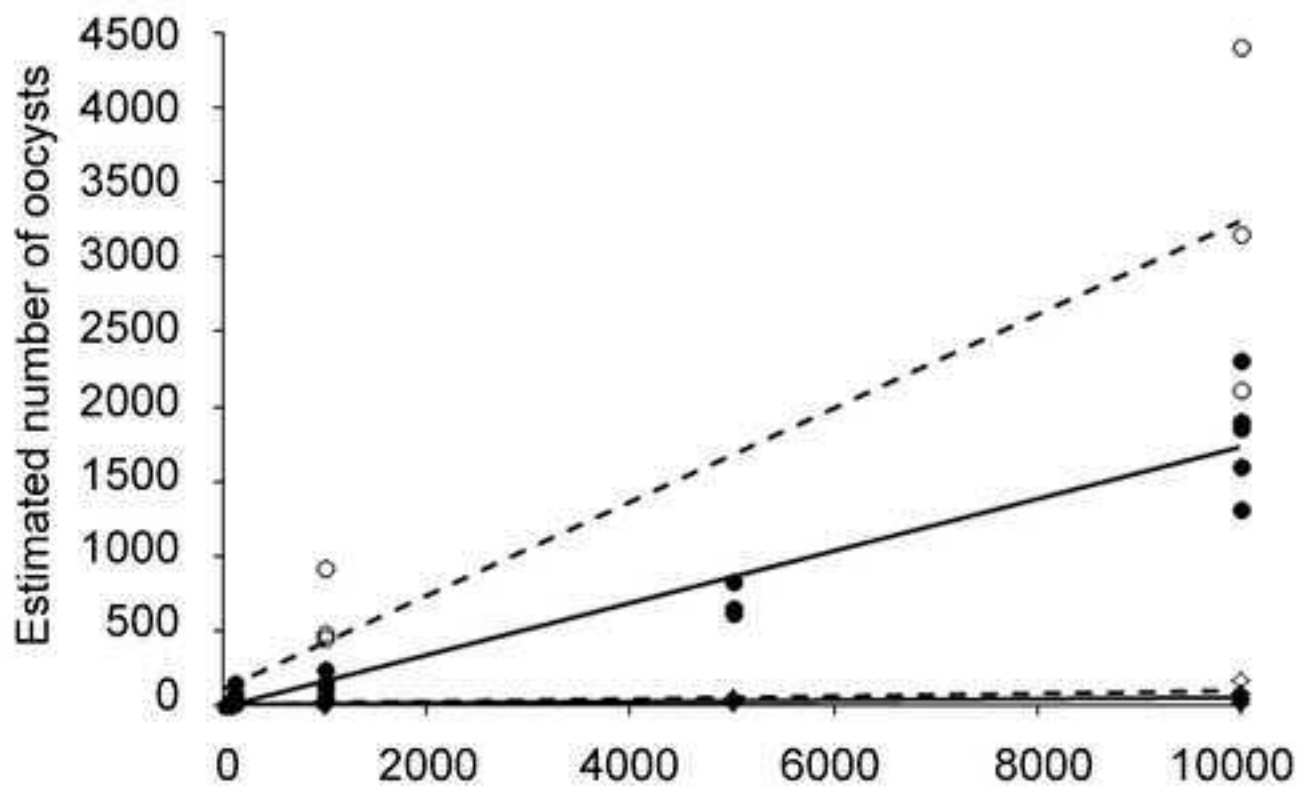


Figure 3

[Click here to download Figure Fig 3.tif](#)

a



b

